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Pathogenic Characteristics of the Korean 2002 Isolate of Foot-and-Mouth Disease Virus Serotype O in Pigs and Cattle

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Summary

Experimental infection of susceptible cattle and pigs showed that the O/SKR/AS/2002 pig strain of foot-and-mouth disease virus (FMDV) causes an infection that is highly virulent and contagious in pigs but very limited in cattle. Pigs directly inoculated with, or exposed to swine infected with, strain O/SKR/AS/2002 showed typical clinical signs, including gross vesicular lesions in mouth and pedal sites. In addition, FMDV was isolated from, and FMDV genomic RNA was detected in, blood, serum, nasal swabs and oesophageal–pharyngeal (OP) fluid early in the course of infection. Antibodies against the non-structural protein (NSP) 3ABC were detected in both directly inoculated and contact pigs, indicating active virus replication. In contrast, the disease in cattle was atypical. After inoculation, lesions were confined to the infection site. A transient viraemia occurred 1 and 2 days after inoculation, and this was followed by the production of antibodies to NSP 3ABC, indicating subclincial infection. No clinical disease was seen, and no antibodies to NSP 3ABC were present in contact cattle. Additionally, no virus or viral nucleic acid was detected in blood, nasal swab and OP fluid samples from contact cattle. Thus, the virus appeared not to be transmitted from infected cattle to contact cattle. In its behaviour in pigs and cattle, strain O/SKR/AS/2002 resembled the porcinophilic FMDV strain of Cathay origin, O/TAW/97. However, the latter, unlike O/SKR/AS/2002, has reduced ability to grow in bovine-derived cells. The porcinophilic character of O/TAW/97 has been attributed to a deletion in the 3A coding region of the viral genome. However, O/SKR/AS/2002 has an intact 3A coding region.

Keywords: cattle; foot-and-mouth disease; Korean FMDV; pig; viral infection

Introduction

Foot-and-mouth disease virus (FMDV), a member of the Aphthovirus genus within the Picornaviridae, is the causative agent of an economically important disease of cloven-hoofed animals, and the single most important constraint to trade in live animals and animal products. FMDV is a positive-stranded RNA virus (~8.5 kb), which occurs as seven distinct serotypes (A, O, C, SAT1, SAT2, SAT3 and Asia 1). The Pan-Asia type O topotype has caused great devastation in Asia in recent years (Sakamoto *et al.*, 2002; Knowles and Samuel, 2003; Feng *et al.*, 2004; Wee *et al.*, 2004).

The Republic of Korea had been free from foot-and-mouth disease (FMD) since 1934 until outbreaks were reported in 2000. These outbreaks occurred in cattle farms only. Eradication, at a cost of $233.6 million, was achieved by slaughter, quarantine, prohibition of livestock transport, and vaccination around infected areas. Korea then remained free from FMD

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until 2002, when a further outbreak on a pig farm occurred, spreading to 14 additional pig farms and one dairy farm, with serious implications for recently resumed pork exports worth $400 million annually.

During the 2002 outbreak, field veterinarians observed severe vesicular lesions in pigs on all farms, but such lesions were described in cows only on one farm. Comparison of genomic sequences of the 2000 and 2002 Pan-Asian isolates (O/SKR/2000 and O/SKR/AS/2002) failed to identify any obvious difference that might have accounted for the difference observed in the pathogenic properties of the two strains (Oem *et al*., 2004). To understand a difference in pathogenicity for pigs and cattle of an FMDV strain, it is necessary to examine infection in each host. The 3A protein of FMDV plays a role in viral virulence, and alteration or deletion of this gene is associated with the reduced ability of some strains of FMDV to cause disease in cattle (Mason *et al*., 2003; Pacheco *et al*., 2003; Grubman and Baxt, 2004). Protein 3A is a viral non-structural (NS) protein containing 153 amino acids and its role in FMDV replication is unclear. While this protein is generally highly conserved in most FMDV strains examined to date, an outbreak of FMD in Taiwan in 1997 was caused by a virus carrying a deletion in 3A (codons 93–102) in addition to a highly mutated region downstream of the deletion (Beard and Mason, 2000). This new strain, a member of the Cathay topotype (Huang *et al*., 2000), caused severe disease in swine but no signs of infection in cattle. It is noteworthy that known 3A-deletions were associated with the attenuation of FMDV strains by egg-adaptation for the development of vaccines for cattle in South America (Giraudo *et al*., 1990). The 3A region of several Asian isolates was recently examined and some were found to harbour a second deletion at codons 133–143 (Knowles *et al*., 2001).

The aim of this study was to investigate the characteristics of FMDV O/SKR/AS/2002 and its pathogenicity for cattle and pigs.

Materials and Methods

FMDV Strains

Strain O/SKR/AS/2002 (Oem *et al*., 2004) was originally isolated in pig kidney (IBRS-2) cells from the epithelium of an infected pig in Anseong (AS) Province, Korea in 2002.

In-vitro *Studies of Strain O/SKR/AS/2002 in Different Cell Lines*

IBRS-2 cells (passage 200), bovine kidney cells (BKLF; passage 125) and fetal bovine kidney cells (FBK; passage 3) were seeded in 96-well cell culture plates, each well receiving 100 µl of a suspension containing 4×10^5 cells/ml. The plates were shaken for 30 s and then incubated at 37°C in a humidified 5% CO_2 incubator. Confluent cells were infected with serial 10-fold (10^{-1}–10^{-8}) dilutions of O/SKR/AS/2002 suspension. Replicates of 10 wells (1st to 10th column) in a 96-well plate were used for each virus dilution (25 µl/well) and the plates were incubated at 37°C in a humidified 5% CO_2 incubator for 60 min. To each well were then added 100 µl of D-MEM medium containing fetal bovine serum 5%, before incubating the plates at 37°C in a humidified 5% CO_2 atmosphere for 3 days. For each plate, the number of wells at each dilution with (+) or without (−) a cytopathogenic effect (CPE) was recorded. The 50% endpoint titre of the virus was determined to calculate the 50% tissue culture infectious dose ($TCID_{50}$).

RNA Isolation and Nucleotide Sequence of the 3A Coding Region

Total RNA was directly extracted from O/SKR/AS/2002-infected IBRS-2 cells (the first passage from the original isolate) with Trizol reagent (Life Technologies, Gaithersburg, MD, USA). It was then used as a template for first-strand synthesis of cDNA, with SuperScript First-Strand Synthesis System for RT-PCR (Life Technologies) and random hexamers as primers. The polymerase chain reaction (PCR) amplification was carried out with AdvanTaq DNA Polymerase (Clontech, Palo Alto, CA, USA) and the specific oligonucleotide pair P797/P799 (5′-TGAAGAGCGG-3′/5′-GCAGGTAAAGTG-3′) encoding the non-structural Δ2C/3A/$B_1B_2B_3$/3C/Δ3D region of O/SKR/AS/2002 (GenBank accession number AY312589). PCR products were purified from agarose gels with Qiagen columns (Qiagen, Valencia, CA, USA) and sequenced directly with selected primers and the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.0 (Applied Biosystems, Foster City, CA, USA), followed by resolution on an ABI3100 sequencer. The sequence data were further analysed with the MacVector™ program.

Animal Experiment

All animal manipulations were performed by procedures approved by the Animal Care and Use Committee of the Plum Island Animal Center. A suspension of strain O/SKR/AS/2002 (10^7 $TCID_{50}$/ml) was used to inoculate one Holstein calf (no. 2), weighing 250–300 kg, at six intradermal lingual sites (0.1 ml per site). This animal was immediately housed in a room with four other Holstein cattle

(nos 1, 3–5) of similar size. One Yorkshire pig (no. 61), weighing 25–30 kg, was infected with the same inoculum in the front heel bulb at eight intradermal sites (40 µl per site). This animal was housed with five other pigs (nos. 61, 63–66) in a room separate from that used for the cattle. For each animal, the clinical signs and lesions were recorded daily. Blood (collected in EDTA-treated tubes), serum, nasal swabs, and oro-pharyngeal (OP) fluid were collected daily from 0 to 14 days post-inoculation (DPI) or days post-exposure (DPE), then once every 7 days for the next 6 weeks (i.e., until day 56 DPI/DPE). Pig 61 was killed by the intravenous administration of sodium pentobarbital (Fatal Plus, Vortech, Dearborn, MI, USA) at 4 DPI, after epidermal lesions had fully developed, for complete necropsy and histopathological examination.

Scoring of Clinical Signs

The clinical scores for cattle were determined as follows: tongue lesion beyond inoculation site = 1; mouth lesion other than tongue = 1; lesion on nostril = 1; one lesion per foot = 1; and two or more lesions per foot = 2. The maximum score for cattle was 11. Clinical scores for pigs were based on the sites containing FMD lesions (vesicular lesions, erosion of epithelium, and blanching of the coronary band). One point was awarded for each affected digit, and for each of three sites (tongue, snout, and lower lip) bearing one or more vesicles. The maximum lesion score for pigs was 19. The scores of each pig were recorded daily until the vesicles at all sites had started to heal (Pacheco *et al.*, 2003).

Histopathology

Pig 61 was killed at 4 DPI and a necropsy was performed. Samples of epidermis at the coronary band of all four digits of all four feet, and samples of snout, lip, tongue, heart, lung, liver, kidney and spleen were immersed in 10% neutral buffered formalin, processed by routine methods and embedded in paraffin wax, and sections (5 µm) were mounted on glass microscope slides, stained with haematoxylin and eosin (HE), ''coverslipped'' and examined.

Isolation of Virus from Blood, Serum, Nasal Swabs and OP Fluid

IBRS-2 cells (passage 193) were seeded in T-25 flasks (Corning Inc., Corning, NY, USA). Confluent cells were infected with 200 µl of EDTA-treated blood, nasal swab samples or OP fluid samples and allowed to adsorb for 60 min at 37°C in a 5% CO_2 incubator. Six milliliter of medium (see above) were added to each flask, which was then examined daily for a CPE, characterized initially by rounded cells, and eventually by the entire destruction of the cell monolayer. In the second passage of virus isolation, 200 µl of fluid from previously inoculated T-25 flasks were added to confluent IBRS-2 monolayers in individual wells of 24-well plates (Corning), which were then examined daily. Based on the appearance of CPE or otherwise, the results were recorded as initial virus isolation positive or negative. For each blood sample, 5 ml were collected in EDTA tubes. Nasal swabs were immersed in 5 ml of Hepes medium containing antibiotic. OP fluid was collected into 10 ml of the same medium and ''vortexed'' vigorously. A 500-µl volume of each OP sample in Hepes medium was transferred into a 1-ml Eppendorf tube for centrifugation in an Eppendorf Centrifuge 5415 (Brinkmann Instruments Inc., Westbury, NY, USA) at 14 000 rpm for 2 min. Supernate (200 µl) was utilized for virus isolation. There were control T-25 flasks (the first passage) and control 24-well plates (the second passage) for each inoculation procedure.

Real-time PCR (RT-PCR) on Serum, Nasal Swabs and OP Fluid

For all samples, RNA was extracted with a MagMax-96 viral RNA isolation kit (Ambion, Austin, TX, USA). Briefly, 25 µl of sample were added to 101 µl of lysis/binding mix (Lysis/Binding solution 50 µl, Poly[A] RNA 1 µl, 100% isopropanol 50 µl) in a 96-well round-bottom microtitre plate (Evergreen Inc., Los Angeles, CA, USA). Next, 20 µl of Beads/Binding Mix (RNA binding beads 4 µl, nuclease free water 4 µl, Lysis/Binding solution 6 µl, and 100% isopropanol 6 µl) were added to each well. The plate was gently shaken on a Titer Plate Shaker (Lab-Line Instruments Inc., Melrose Park, IL, USA) at dial position 5 for 4 min. Beads were pelleted for 2 min on a 96-well magnet (Ambion) and the supernate was removed. To each well, 100 µl of Wash Solution I Mix (Wash Solution I [Ambion] 50 µl, 100% isopropanol 50 µl) were added, and the plate was shaken at dial position 5 for 30 s. The beads were then pelleted (1 min) and the supernate was removed. The beads were washed twice with 100 µl of Wash Solution II Mix per well (Wash Solution II [Ambion] 30 µl, 100% ethanol 180 µl), shaken at dial position 5 for 30 s, and the supernate was removed after pelleting the beads for 30 s. The beads were shaken vigorously at dial position 9 for 2 min to dry them. They were then mixed with Elution Solution (Ambion) 25 µl/well and shaken at dial position 9 for 4 min. Finally, the beads were pelleted for 2 min

and the RNA was transferred to a storage plate and frozen at −70°C.

Reverse transcriptase real-time PCR (rRT-PCR) was performed on the extracted RNA. Primers and probe targeting the 3D region of FMDV were designed previously (Callahan *et al*., 2002). Reagents from the EZ rTth Kit (Applied Biosystems, Foster City, CA, USA) were used to prepare the master mix according to the guidelines of the manufacturer for individual component concentrations. The final rRT-PCR mixture (25 µl per sample) consisted of the 5× buffer solution provided in the kit, with the addition of 25 mM MnOAc, a 0.2 µM concentration of each primer and 0.1 µM concentration of probe, 1.2 mM dNTPs, 2.5 U rTth polymerase, and 50× Rox reference dye (Invitrogen, Carlsbad, CA, USA) 0.031 µl. For testing, 22.5 µl of master mix and 2.5 µl of RNA sample were added to 96-well (0.2 ml) polypropylene plates (Stratagene, La Jolla, CA, USA). The rRT-PCR was performed on the Stratagene Mx4000 thermocycler as follows: an initial step of 60°C for 600 s, followed by 45 amplification cycles of 95°C for 30 s and 65°C for 60 s (Callahan *et al*., 2002)

Measurement of Non-structural Protein (NSP) Antibodies

The 3ABC indirect enzyme-linked immunosorbent assay (I-ELISA) was modified from Meyer *et al*. (1997). Briefly, 3ABC protein was produced in a baculovirus system. Crude 3ABC antigen and negative control antigen in 8 M urea were coated in triplicate on immulon 2 plates (Dynex Technologies, Chantilly, VA, USA) for a minimum of 24 h at 4°C. Test serum was diluted 1 in 100 in PBST (phosphate-buffered saline containing Tween-20 0.05%) and applied to the washed plate for 30 min. Antibody binding was detected by applying protein A/G peroxidase (Pierce, Rockford, IL, USA) diluted in PBST, followed by addition of tetramethyl benzidine (TMB) and substrate. Reactions were stopped by addition of 1 M sulphuric acid. To validate the test, four standard sera from FMDV convalescent animals with known percentage arbitrary values were run with each set of plates to determine the linear regression. For four standard sera in each run, the arbitrary value *vs* absorbance should be linear; $r^2 > 0.92$ for a valid test. For all samples, the values from the negative control antigen wells were subtracted from the values from the wells containing 3ABC protein. The OD values of tested serum samples were automatically converted into arbitrary values by a linear equation (Softmax Pro Program; Molecular Devices, Sunnyvale, CA, USA). Sera from an FMDV-infected and a non-infected animal were run on each plate as positive and negative controls, respectively. The arbitrary cut-off value of this test was 10 in cattle and 13 in pigs.

Results

Replication of Strain O/SKR/AS/2002 in Different Cell Lines

CPEs were produced in all three cell lines (BKLF, FBK and IBRS-2). The $TCID_{50}$ values of the same stock of virus (first passage in IBRS-2 cells of the original isolate) in BKLF, FBK and IBRS-2 cells were $10^{7.3}$, $10^{6.0}$ and $10^{6.2}$, respectively, i.e., approximately one log higher in BKLF cells than in IBRS-2 or FBK cells. No CPE was observed in uninfected control cells.

Comparison of 3A Sequences

To map changes in the NSP 3A, RNA was isolated from FMDV-infected IBRS-2 cells and subjected to RT-PCR. The PCR was designed to amplify a 3412-bp region consisting of a fragment of 2C, all of 3A/$3B_1B_2B_3$/$3C^{pro}$, and a partial sequence of $3D^{pol}$. The PCR product was extracted from the agarose gel and sequenced. The sequence data showed no deletions in 3A and accorded with a recently deposited sequence of the L-fragment of O/SKR/2002 sequence (GenBank accession number AY312589), except for one amino-acid change ($T_{116}A$) found in codon 116 within the hypervariable region of FMDV 3A.

Clinical Signs and Lesion Score

Cattle. At 1 DPI, calf 2 showed increased salivation and blanching in five of the six intradermal lingual sites of inoculation. At 2 DPI, salivation was still present, erosions had started to develop on the five affected lingual sites, and the rectal temperature had reached 39.4°C. At 3 DPI, five discrete, oval, green lesions (indicative of epithelial necrosis and shallow erosion) were observed on the tongue (Fig. 1), corresponding to virus injection sites, and the rectal temperature was 39.1°C. At 4 DPI, the erosions persisted but the rectal temperature had decreased to 38.7°C. No vesicle was observed on any externally visible epithelium of this animal throughout the experiment. The injection site lesions were fully healed by 8 DPI. None of the contact cattle (nos 1, 3—5) showed any clinical signs or vesicular lesions throughout the experiment.

Pigs. At 1 DPI, the inoculated pig (no. 61) showed blanching of all eight injection sites in the heel bulb. During day 2, vesicles started to develop (1) at the injection sites, (2) on the right edge of the tongue, and (3) on all feet, including the coronary bands, heel bulbs, and interdigital spaces. At 4 DPI, rupture of vesicles and epithelial necrosis on the heel bulbs

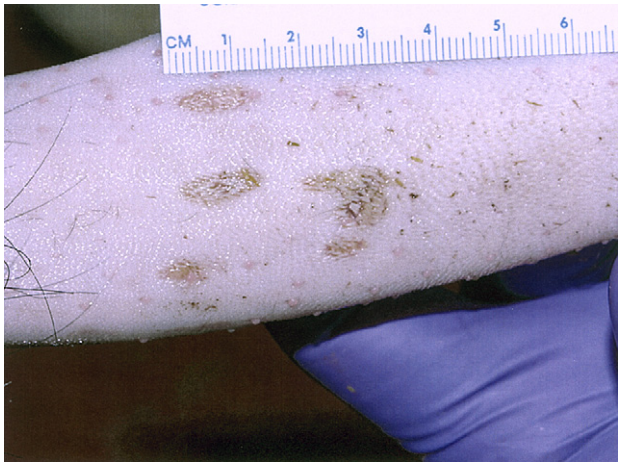

Fig. 1. Tongue of calf 2 at 3 DPI. Five discrete foci of coagulative necrosis are seen, with mild superficial erosion of the epithelium. The lesions, which were confined to intradermolingual injection sites, were completely resolved by 8 DPI.

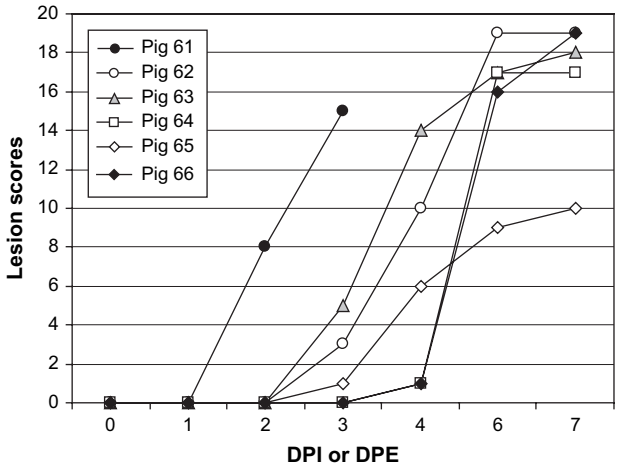

Fig. 3. The lesion scores of pigs 61–66 on various days post-inoculation or post-exposure (DPI or DPE).

were observed (Fig. 2). At 3 DPI, the erosion score of pig 61 had increased to 15 (Fig. 3), and at 4 DPI this animal was killed. Three contact pigs (62, 63 and 65) showed foot lesions at 3 DPE and the remainder (64 and 66) at 4 DPE. At 7 DPE, the lesion scores for contact pigs 62, 63, 64 and 66 had reached 19, 18, 17 and 19, respectively. Pig 65 had a lesion score of 10 at 7 DPI (Fig. 3). All contact pigs were pyrexic from 4 to 6 DPE, the temperature at 6 DPE being >40°C.

Histopathological Findings in Pig 61 at 4 DPI

Coronary band and digits of all four feet. Microscopical changes observed in haired skin sections of the feet (also snout and lip; see below) included moderate to marked acute vesicular, erosive and ulcerative dermatitis, with epithelial degeneration and necrosis, intraepithelial microabscesses, oedema, and secondary bacterial infection. Individual and multifocal microvesicles and large coalescing vesicles, some in the early stages of rupturing, were observed. There were multiple epidermal erosions and ulcerations, some of which resulted in colonization of exposed dermal collagen bundles by coccal bacteria. In all sections, there was degeneration, coagulative necrosis and dissociation of epithelial cells (individual cells and aggregates) within the stratum spinosum, characterized by shrunken hypereosinophilic cytoplasm, condensation of chromatin, and pyknosis. Perinuclear cytoplasmic vacuolation (intracellular oedema) of epithelial cells at the margins of the lesions was often noted, as also was infiltration of the epidermis by viable and degenerate neutrophils, with formation of micropustules. Small intact vesicles contained homogeneous eosinophilic material (proteinaceous fluid); larger vesicles consisted of rarefied areas containing large numbers of neutrophils, smaller numbers of macrophages, and variable amounts of eosinophilic fibrillar material (fibrin) (Fig. 4). Spinocytes (individual cells and rafts) could be seen desquamating into the lumina of many vesicles. Within areas of epithelial erosion and ulceration, the adjacent intact epidermis and subjacent dermis contained large amounts of viable and degenerate neutrophils, and pyknotic and karyorrhectic debris. The dermis was often infiltrated in a multifocal and perivascular pattern by moderate numbers of macrophages admixed with smaller numbers of neutrophils and lymphocytes. Endothelial cell hypertrophy was noted occasionally, characterized by plump endothelial cell nuclei protruding into the vascular lumen. There was often extensive rarefaction and expansion of the papillary dermis, seen as a clear space (oedema); this change occasionally extended into the reticular dermis (Fig. 5).

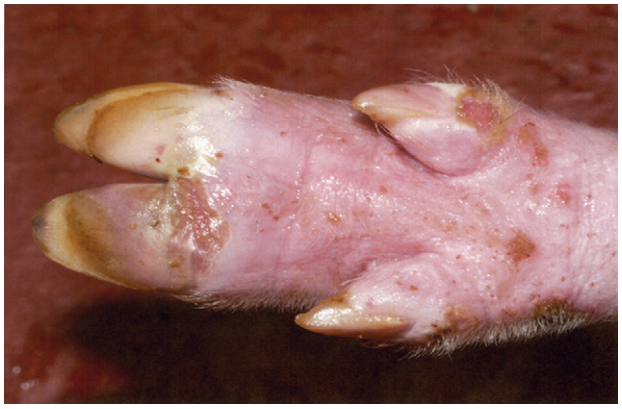

Fig. 2. Epithelial necrosis on heel bulbs of the left hind foot of pig 61 at 4 DPI.

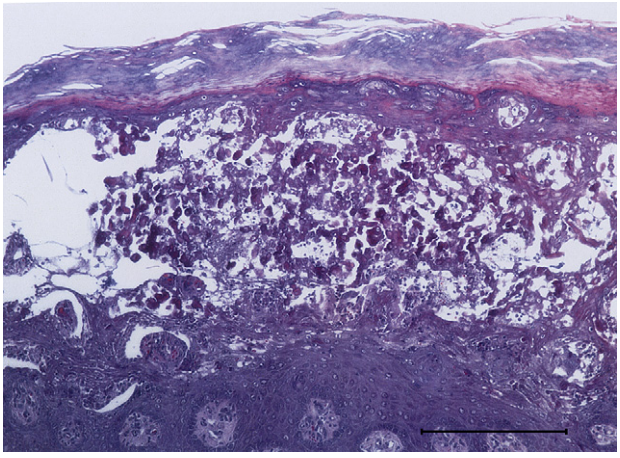

Fig. 4. Coronary band (fourth digit, left thoracic limb) of pig 61 at 6 DPI. Multiple small vesicles coalesce to form a large intact vesicle within the stratum spinosum. Degenerating epithelial cells, with angular and hypereosinophilic cytoplasm and pyknotic nuclei, are detaching and accumulating within vesicle fluid, admixed with strands of fibrin and small numbers of neutrophils. Note microvesicle at dorsal right aspect of the larger vesicle, as well as epithelial cells with hydropic degeneration and perinuclear oedema. HE. Bar, 250 μm.

Snout. In one section of snout there was necrosis of epithelial cells in the superficial stratum spinosum, with rounding and dissociation of cells. In another section, within the same region of the epidermis, a moderately sized intact vesicle was present, partly filled by neutrophils, lymphocytes, proteinaceous fluid, fibrin and a few erythrocytes. Increased clear space between epithelial cells (spongiosis) and intracellular oedema was noted adjacent to the vesicle.

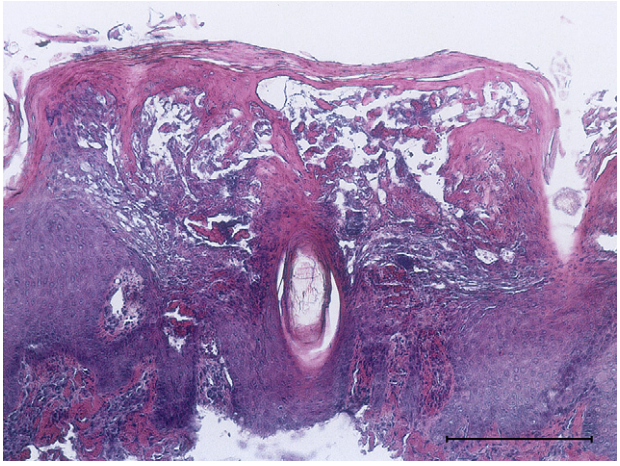

Fig. 5. Coronary band (fourth digit, right pelvic limb) of pig 61 at 6 DPI. Multiple intact vesicles in the stratum spinosum are partly separated by a cord of epithelial cells. Note detachment of degenerate epithelial cells (individual and rafts), with hypereosinophilic angular cytoplasm and hyperchromatic nuclei. Note intracytoplasmic oedema (ballooning degeneration) in intact epithelial cells immediately to the left of the vesicles. HE. Bar, 250 μm.

Lip. There was an extensive ruptured vesicle within the haired skin of the lip, with formation of a large flap of necrotic epithelium. Multiple discrete and coalescing epidermal micropustules were also present, as was extensive intracellular and intercellular oedema. Hydropic degeneration of epithelial cells of hair follicles was seen occasionally. Rarefaction of the dermis and dilatation of dermal lymphatic vessels (oedema) accompanied mild lymphohistiocytic infiltrates. No alterations were noted in the non-keratinizing mucosal epithelium.

Heart. In a section of the left ventricle, mild multifocal lymphohistiocytic myocarditis was observed, characterized by scattered small aggregates of lymphocytes and histiocytes within the myocardial interstitium and in the subendocardial connective tissue (Fig. 6). Mild cardiac myofibre degeneration, characterized by granular fragmentation of the sarcoplasm, was also noted.

Other tissues. No significant microscopical lesions were noted in sections of the tongue, lung, liver, kidney or spleen.

Virus Isolation (Cattle and Pigs)

In calf 2, FMDV was first isolated from the whole blood at 1 DPI (Table 1). Two days later it was also isolated from OP fluid, but no virus was isolated from nasal swab samples. In contact cattle, no virus was isolated from blood, nasal swab or OP fluid samples collected at any timepoint. In pig 61, FMDV was

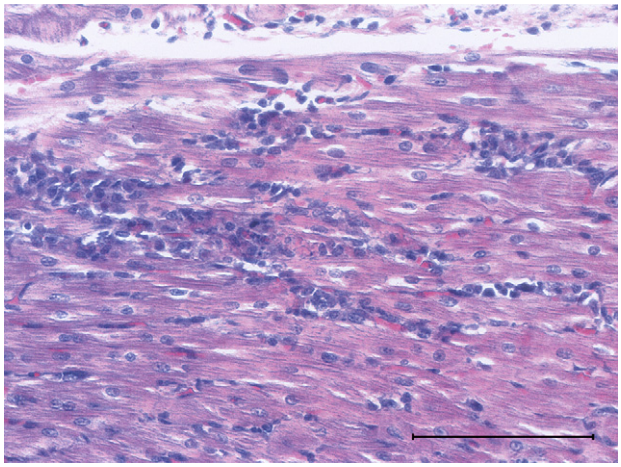

Fig. 6. Heart, pig 61, at 6 DPI. Moderate numbers of histiocytes and lymphocytes infiltrate the myocardial interstitium. HE. Bar, 100 μm.

Table 1
Virus isolation (VI) from EDTA-treated blood and real-time polymerase chain reaction (PCR) of serum samples collected on various days post-inoculation (DPI) or days post-exposure (DPE) from cattle and pigs

DPI or DPE	Calf 2*		Calf 1		Calf 3		Calf 4		Calf 5		Pig 61*		Pig 62		Pig 63		Pig 64		Pig 65		Pig 66	
	VI†	*PCR*	*VI*	*PCR*	*VI*	*PCR*	*VI*	*PCR*	*VI*	*PCR*	*VI*	*PCR*	*VI*	*PCR*	*VI*	*PCR*	*VI*	*PCR*	*VI*	*PCR*	*VI*	*PCR*
0	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−
1	−/+	−	−/−	−	−/−	−	−/−	−	−/−	−	+/+	−	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−
2	−/+	−	−/−	−	−/−	−	−/−	−	−/−	−	+/+	+	−/−	−	−/+	−	−/−	−	−/−	−	−/−	−
3	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	+/+	+	+/+	−	+/+	+	−/−	−	−/−	−	−/−	−
4	−/−	+	−/−	−	−/−	−	−/−	−	−/−	+	⋮	‡+	+/+	+	+/+	+	−/−	−	−/−	+	−/+	−
6	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	⋮	⋮	−/−	−	−/−	−	+/+	+	−/−	−	−/+	+
8	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	⋮	⋮	−/−	+	−/−	−	−/−	−	−/−	−	−/−	−
10	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	⋮	⋮	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−
14	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	⋮	⋮	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−
28	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	⋮	⋮	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−
56	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	⋮	⋮	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−

*Calf 2 and pig 61 were inoculated with strain O/SKR/AS/2002.
†The VI results are expressed as first passage/second passage in IBRS-2 cells, and as FMDV isolated (+) or not isolated (−).
‡Pig 61 was killed for necropsy and histopathological examination at 4 DPI, i.e., when epidermal lesions were fully developed.

isolated from blood at 1 DPI, and the viraemia persisted for an additional 2 days before the animal was killed; virus was also isolated from nasal swab and OP fluid samples from this donor pig at 2–3 DPI. FMD virus was isolated from blood, nasal swab, and OP fluid samples of various contact pigs from 2 to 6 DPE. No virus was isolated from blood or nasal swabs of pig 65, or from OP fluid of pig 64 (Tables 1–3).

Real-time PCR (Cattle and Pigs)

The inoculated calf (no. 2) gave positive results with blood collected at 4 DPI and with OP fluid samples collected at 2–6 DPI. Contact cattle gave negative results with all serum, nasal swab, and OP fluid samples. The inoculated pig (no. 61) gave positive results with blood, nasal swab and OP fluid samples collected at 2–3 DPI. Contacts gave positive results with blood, nasal swab and OP fluid samples from 3 to 8 DPE, 2 to 8 DPE, and 2 to 14 DPE, respectively (Tables 1–3).

NSP 3ABC Antibody Test

Calf 2 developed antibodies to 3ABC (detectable by I-ELISA) at 8 DPI; the titre reached a peak at 28 DPI and then started to decline. No 3ABC antibodies were detected in any of the contact cattle from 0 to 56 DPE. The 3ABC antibodies of pigs 62, 63, and 64 increased significantly at 8, 9, and 11 DPE, respectively, and all reached a peak at 14 DPE before starting to decline. The 3ABC antibodies of pig 66 showed a significant increase at 11 DPE and reached a peak at 21 DPE before declining. Pigs 62–64 and 66 had antibody titres over 100% arbitrary values at their maximum; however, pig 65 showed a significant increase in 3ABC antibody titre at 13 DPE but did not reach 40% arbitrary values even with the highest titre at 21 DPE (Figs. 7 and 8).

Discussion

The experiment showed that strain O/SKR/AS/2002 was highly virulent and contagious for pigs, causing vesicles in the mouth and on the feet. Active viral replication in all six infected pigs (one inoculated and five in-contact) was demonstrated by (1) isolation of FMDV in cell culture from whole blood, nasal swab and OP fluid samples, (2) the presence of FMDV nucleic acid in serum, nasal swabs and OP fluid, (3) the presence of gross lesions consistent with FMD, and (4) the presence of antibodies against the NS protein 3ABC in serum.

In contrast, the disease in cattle was atypical, no lesions developing in inoculated or in-contact cattle, other than at the injection sites in the single

Table 2
Virus isolation (VI) and real-time polymerase chain reaction (PCR) of nasal swabs collected on various days post-inoculation (DPI) or days post-exposure (DPE) from cattle and pigs

DPI or DPE	Calf 2*		Calf 1		Calf 3		Calf 4		Calf 5		Pig 61*		Pig 62		Pig 63		Pig 64		Pig 65		Pig 66	
	VI†	*PCR*	*VI*	*PCR*	*VI*	*PCR*	*VI*	*PCR*	*VI*	*PCR*	*VI*	*PCR*	*VI*	*PCR*	*VI*	*PCR*	*VI*	*PCR*	*VI*	*PCR*	*VI*	*PCR*
0	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−
1	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−
2	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	+/+	+	+/+	+	+/+	+	−/−	+	−/−	−	−/−	+
3	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	+/+	+	+/+	+	+/+	+	+/+	+	−/−	+	+/+	+
4	−/−	−	−/−	−	−/−	−	−/−	−	−/−	+	…‡		+/+	+	+/+	+	−/−	−	−/−	+	−/+	+
6	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	…		+/+	+	+/+	+	−/+	+	−/−	+	+/+	+
8	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	…		−/−	−	−/−	−	−/−	+	−/−	−	−/−	−
10	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	…		−/−	−	−/−	−	−/−	−	−/−	−	−/−	−
14	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	…		−/−	−	−/−	−	−/−	−	−/−	−	−/−	−
28	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	…		−/−	−	−/−	−	−/−	−	−/−	−	−/−	−
56	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	…		−/−	−	−/−	−	−/−	−	−/−	−	−/−	−

*Calf 2 and pig 61 were inoculated with strain O/SKR/AS/2002.
†The VI results are expressed as first passage/second passage in IBRS-2 cells, and as FMDV isolated (+) or not isolated (−).
‡Pig 61 was killed for necropsy and histopathological examination at 4 DPI, i.e., when epidermal lesions were fully developed.

Table 3
Virus isolation (VI) and real-time polymerase chain reaction (PCR) of OP fluid samples collected on various days post-inoculation (DPI) or days post-exposure (DPE) from cattle and pigs

DPI or DPE	Calf 2*		Calf 1		Calf 3		Calf 4		Calf 5		Pig 61*		Pig 62		Pig 63		Pig 64		Pig 65		Pig 66	
	VI†	*PCR*	*VI*	*PCR*	*VI*	*PCR*	*VI*	*PCR*	*VI*	*PCR*	*VI*	*PCR*	*VI*	*PCR*	*VI*	*PCR*	*VI*	*PCR*	*VI*	*PCR*	*VI*	*PCR*
0	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−
2	−/−	+	−/−	−	−/−	−	−/−	−	−/−	−	+/+	+	−/−	+	−/−	+	−/−	+	−/−	+	+/+	+
4	+/+	+	−/−	−	−/−	−	−/−	−	−/−	+	…‡		+/+	+	+/+	+	−/−	+	−/−	−	+/+	+
6	−/−	+	−/−	−	−/−	−	−/−	−	−/−	−	…		−/−	+	−/−	+	−/−	+	+/+	+	−/−	+
8	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	…		−/−	−	−/−	+	−/−	+	−/−	+	−/−	+
10	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	…		−/−	−	−/−	−	−/−	+	−/−	−	−/−	+
14	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	…		−/−	−	−/−	−	−/−	−	−/−	+	−/−	−
28	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	…		−/−	−	−/−	−	−/−	−	−/−	−	−/−	−
56	−/−	−	−/−	−	−/−	−	−/−	−	−/−	−	…		−/−	−	−/−	−	−/−	−	−/−	−	−/−	−

*Calf 2 and pig 61 were inoculated with strain O/SKR/AS/2002.
†The VI results are expressed as first passage/second passage in IBRS-2 cells, and as FMDV isolated (+) or not isolated (−).
‡Pig 61 was killed for necropsy and histopathological examination at 4 DPI, i.e., when epidermal lesions were fully developed.

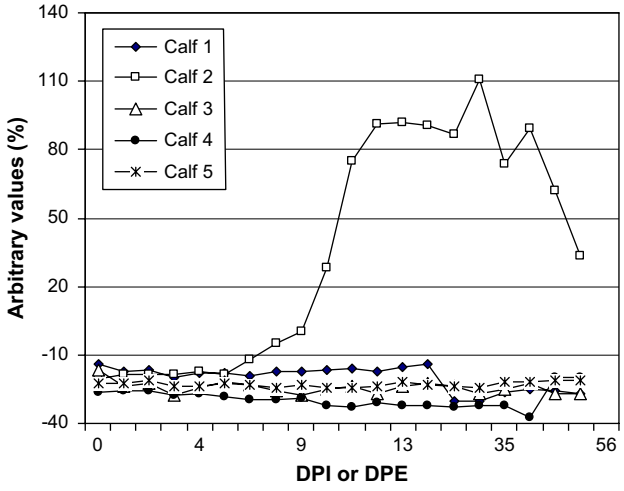

Fig. 7. The 3ABC (NSP) antibodies of calfs 1–5 on various days DPI or DPE.

inoculated animal (calf 2). Although 3ABC antibodies were detected in calf 2, the only lesions that occurred were erosions at the injection sites on the tongue. These were accompanied by weak viraemia. In the contact cattle, neither 3ABC antibodies nor vesicles occurred; furthermore, no virus could be isolated from whole blood, nasal swabs or OP fluid samples and PCR testing of serum, nasal swabs and OP fluid samples gave negative results. Thus, the experiment demonstrated failure of this virus to spread to the contact cattle over an 8-week period.

Strain O/SKR/AS/2002 is phenotypically similar to the porcinophilic strain (O/TAW/97) of Cathay virus. *In-vivo* studies of FMDV strain O/TAW/97 performed at the OIE World Reference Laboratory for FMD at Pirbright, UK showed that this virus did not cause clinical disease in cattle even by direct inoculation in the tongue (Dunn and Donaldson, 1997).

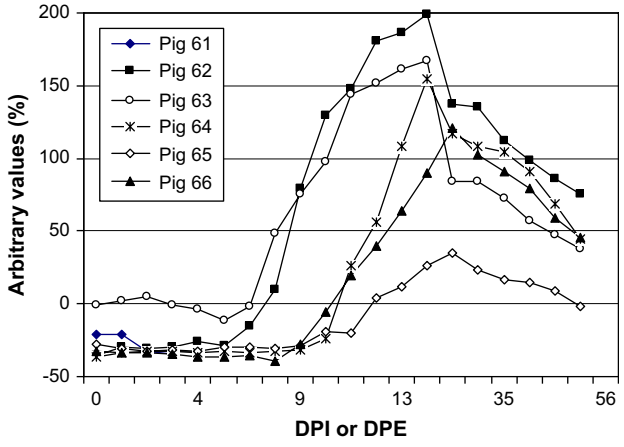

Fig. 8. The 3ABC (NSP) antibodies of pigs 61–66 on various days DPI or DPE.

In-vitro studies of O/TAW/97 demonstrated reduced ability to grow in bovine cell cultures (Giraudo *et al.*, 1987; Sagedahl *et al.*, 1987; Beard and Mason, 2000; Mason *et al.*, 2003); however, O/SKR/AS/2002 grew efficiently in both bovine and porcine cells. The apparent inability of strain O/TAW/97 to replicate effectively in cattle is similar to that of Cathay lineage type O FMDVs co-circulating in Asia. The question arises as to whether the O/SKR/AS/2002 isolate sent to the Plum Island Animal Disease Center was related to the Cathay lineage of viruses and not to the Pan-Asia lineage. One of the genetic markers that distinguishes the two lineages can be found in the 3A viral protein sequence. Cathay viruses, which show restricted growth in cattle, possess a deletion in 3A protein (Sagedahl *et al.*, 1987; Giraudo *et al.*, 1990; Dunn and Donaldson, 1997; Yang *et al.*, 1999; Beard and Mason, 2000; Huang *et al.*, 2000; Grubman and Baxt, 2004). No such deletions are present in type O Pan-Asia isolates. Our sequence data, and a recently deposited sequence of the L-fragment of O/SKR/AS/2002, show no deletions in 3A. However, there is one amino-acid difference in codon 116 within the hypervariable region of FMDV 3A. One possible explanation for the difference is that the sequence deposited at GenBank was an original isolate and the O/SKR/AS/2002 we used for sequencing had been passaged once in IBRS-2 cells. The results, including the mild disease produced in the cattle, are consistent with the virulence of the Pan-Asia topotype and further indicate that the 3A deletion is not the only genetic marker related to host-range restriction.

The pathological findings in pig 61 were similar to those reported by Alexandersen *et al.* (2003). Although no gross lesions were observed in the heart, mild multifocal lymphohistiocytic myocarditis in a section of the left ventricle and mild cardiac myofibre degeneration were seen microscopically. In contact pigs, the occurrence of virus in OP fluid and nasal mucosa was followed by viraemia (Tables 1–3). Some studies have suggested that the initial site of FMDV replication is the pharynx (Burrows *et al.*, 1981; Zhang and Kitching, 2001; Alexandersen *et al.*, 2003) or in the respiratory tract (Brown *et al.*, 1996). In the present study, viraemia generally accorded with lesion scores in pigs. After 6 DPI or DPE, no live virus was detected in blood, nasal swab or OP fluid samples. After 14 DPI or DPE, for all animals, no viral nucleic acid was detected in blood, nasal swabs or OP fluid. Alexandersen *et al.* (2003) reported that host immunity to FMD was primarily mediated by circulating antibodies, and that progressive clearance of virus from the circulation and a significant reduction from most organs through excretions and secretions were evident; in addition,

virus isolation in BTY cells and quantitative RT-PCR consistently failed to show that FMDV persisted in pigs for more than 3–4 weeks.

In this study, the combination of 96-well RNA extraction techniques and real-time PCR assisted in the rapid handling and processing of samples. It proved more efficient than the traditional RNA extraction procedure and RT-PCR, especially for dealing with large numbers of samples. RNA was extracted from sera, because a procedure for extracting RNA from blood on a 96-well plate had not yet been developed. The number of positive results obtained by virus isolation from EDTA-treated blood was greater than the number found by the use of PCR on serum samples. Possibly, FMDV may be cell-associated and therefore lost during serum preparation. It is also possible that serum samples contain an inhibitor of reverse transcriptase. Quan *et al*. (2004) reported that the sensitivity of FMDV isolation was 10–100 times less than that of RT-PCR. In the present study, the lack of sensitivity of FMDV isolation in cell culture may be similarly explicable. The results suggest that in pigs, nasal swab samples are preferable to EDTA-treated blood and OP fluid samples for virus isolation (Tables 1–3). Recent evidence suggests that viral replication is greater in the mucosa of pigs than in the lung (Oleksiewicz *et al*., 2001). In the present study, FMDV nucleic acid was detected for a longer period from porcine OP fluid samples by real-time PCR (14 DPE), than from serum or nasal samples (8 DPE).

In the 2002 Korean outbreak, two FMDV isolates (one from pigs and the other from cattle) of strain O/SKR/AS/2002 (Oem *et al*., 2004) appeared to be nearly identical on sequencing (data not shown). The strain used in the present experiment was from a pig. The data from 3A sequencing of pig-derived O/SKR/AS/2002 do not explain why this virus, which is highly infectious for pigs, has restricted growth properties in cattle. It is possible that more than one factor determines the porcinophilic character of this strain.

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